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DEVELOPMENT OF CHLOROPLAST MICROSATELLITE (CPSSR) MARKERS FOR THE GENUS JATROPHA

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ABSTRACT

Physic nut or Jatrophacurcas is the only one which is an attractive species in Genus Jatrophaas an alternative to biodiesel. The big problems of J. curcas are their low production, non - homogenous fruit ripening and low genetic diversity. Therefore, this research focused on developing 14 chloroplast microsatellite (cp SSR) markers for genetic diversity study of 17 Jatropha accessions including 12 of Jatrophacurcas and 5 of the other species. However, there are only 4 primers i. e., JaCpSSR_2, JaCpSSR_5, JaCpSSR_10 and JaCpSSR_11 which generated polymorphic bands and produced 25 alleles by fragment analysis. The dendrogram was constructed by NTSYSpc-2.20k, UPGMA method and the similarity index ranged from 0.68-1.00 which separated the samples into 3 groups. The group I consisted of all samples of J. curcas which accessions from Lampang was closely related to Chiangmai and Loey. Similarly, J. curcas from Lamphun and Khonkaen with the similarity index was 1.00. Group II was J. integerrima (1), J. integerrima (2), J. multifida and J. gossypifolia formed a group together and group III was J. podagrica.

KEYWORDS: Genus Jatropha, SSR Markers & Chloroplast

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Original Article

INTRODUCTION

Physic nut or *Jatrophacurcas* is the flowering plant in Euphorbiaceae family. *J. curcas* is a tropical species native to Mexico and Central America. Recently, it is broadly distributed in Latin America, India and South-East Asia. There are approximately 470 species in *Jatropha* species. In case of Thailand, there are only 5 species, *J. gossypifolia*, *J. podagrica*, *J. integerrima*, *J. multifida* and *J. curcas*[1]. *J. Caracas* has potential to be vigorous, drought and pest-tolerant. Actually, *J. curcas* can be easily cultivated under climatic conditions in Thailand. The seeds contain approximately 30-40% oil that can convert to biodiesel by transesterification and can be used in low speed engine immediately [2]. However, the difference time of pollen grains and stigma development make non-homogenous fruit ripening [3]. Thus, the low production and low genetic diversity are still problematic for increasing the seed oil yield. Therefore, the well understanding in a genetic relationship in genus *Jatropha* is important for efficient bleeding, management and utilization [4].

Previously, there are the study of the variation by using RAPD and RFLP but the diversity among intra- and interspecific of *Jatropha* species are very low [5]. Microsatellites or simple sequence repeats (SSRs) are tandem repeats of 1-6 nucleotides which are distributed throughout the genomes. SSRs are the markers of choice for evaluating the genetic diversity and a variety of plant species because of their metallic, higher levels of polymorphism, codominant inheritance and relatively abundant [6]. Microsatelliite markers have been successfully identified in a number of plants such as *Loliumperenne* or rye-grass. These markers were used for characterizing the genetic diversity in *Lolium* and different grass species. The *L. multifolum* could separate from other *Lolium* and

found the indel (insertion/deletion) [7]. Microsatelliite markers have been successfully used for population and evolutionary studies for cultivar development, but they are not proper for phylogenetic studies above the species level. In chloroplast DNA varies in terms of haploid, uniparental inheritance and lack of recombination compared with the nuclear genome [8]. Chloroplast simple sequence repeat (cp SSR) in noncoding regions shows higher sequence variation than coding regions. In this study, chloroplast simple sequence repeat (cp SSR) markers were developed from the complete chloroplast genome of *J. curcas* and studied the genetic diversity of *Jatropha* in Thailand.

MATERIALS AND METHODS

Plant Materials

In total, 17 samples of young leaf *Jatropha* were obtained from Nakhonratchasima crop research center and Reseach and Practice Garden of Department of Agricultural Science, Faculty of Agriculture Natural Resources and Environment, Naresuan University (Table 1). Twelve cultivars consist of *Jatrophacurcas* Chiang Mai, Loey, South Africa, Nakhonratchasima, Lamphun, Lampang, Myanmar, Khonkaen, Nakonsawan, Phitsanulok and Mexico. In addition, *J. gossypifolia, J. podagrica, J. interrima and J. multifida*werecollected many areas in Thailand.

Table 1: List of 17 *Jatropha* Accessions Included in this Study and the Location where the Accession was Collected

Accession Number	Species	Location
Ja1	*Jatrophacurcas*	Chiang Mai
Ja2	*Jatrophacurcas*	Loey
Ja3	*Jatrophacurcas*	Repubic of South Africa
Ja4	*Jatrophacurcas*	Chiang Mai
Ja5	*Jatrophacurcas*	Lamphun
Ja6	*Jatrophacurcas*	Lampang
Ja7	*Jatrophacurcas*	Myanmar
Ja8	*Jatrophacurcas*	Khonkaen
Ja9	*Jatrophacurcas*	Nakhonsawan
Ja10	*Jatrophacurcas*	Phitsanulok
Ja11	*Jatrophacurcas*	Maxico(non-toxic5)
Ja12	*Jatrophacurcas*	Maxico (non-toxic6)
Ja13	*Jatrophagossypifolia*(1)	Phichit
Ja14	*Jatrophagossypifolia* (2)	Phetchaburi
Ja15	*Jatrophaintegerrima*	phitsanulok
Ja16	*Jatrophamultifida*	Phitsanulok
Ja17	*Jatrophapodagrica*.	Nakhonratchasrima

DNA Extraction

Total DNA was extracted by modifyingDoyle & Doyle, (1990) CTAB method [9]. Young leaves (0.5 g) were ground to a fine powder in liquid nitrogen. The powder was placed in 3000 µl of 2% cetyltrim ethyl ammonium bromide (CTAB) extraction buffer with modification. Furthermore, the solution was vortexed and incubated 65°C for 1 hour with inverting the tube every 15 minutes. Next, 1 volume of chloroform: isoamyl alcohol (24:1) was added to the solution, which was inverted gently and centrifuged at 6000 rpm for 15 minutes. The supernatant was transferred to new 1.5 ml micro centrifuge tube. This step was repeated once. Then, 0.1 volume of 3M Sodium acetate and 2 volumes of absolute ethanol were added to the supernatant for DNA precipitation. Samples were gently mixed by inversion and kept at -20°C for 30 minutes. After that, the mixture was centrifuged at 6000 rpm for 5 minutes. The liquid phase was discarded and washed the DNA pellet by 500 µl of 70 % ethanol. The pellet was set to dry and re suspended in 100 µl deionized water

with RNase (1µg/ml) 3 µl. The solution was then incubated at 37°C for 1 hr., and after storing at -20°C. Genomic DNA was quantified in 0.8% agarose electrophoresis and visualized using ethidium bromide staining.

Chloroplast SSR Primers Design

The 163,856 bp Genus *Jatropha* chloroplast genome DNA sequence was downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) [10] and saved in Fasta for genomic DNA. Simple sequence repeats identification tool software (SSIT) (http://archive.gramene.org/db/markers/ssrtool) [11] were used to search for microsatellite tandem repeats in the genome sequence data. Primers were designed using franking regions between microsatellite sequence. The general guideline to design primers were defined to avoid secondary structure such as hairpin loop, self-complementary and primer-dimer using Oligo Calc [12] and sent to Macrogen company for sequencing.

Chloroplast SSR Analysis

Fourteen chloroplast SSR primers were performed by Polymerase Chain Reactions in a total volume of 25 µl containing 100 mg DNA, 0.2 mMd NTP, 2.5 µM $MgCl_2$, 0.75 U *Taq* polymerase (RBC Bioscience) and 0.2 µM of each primer pair. The amplification reaction conditions were as follows: 3 min Pre-denaturation step at 94°Cfollowed by thirty cycles of 30 s at 94°C, Annealing temperature depend on each primer for 30 s, 45 s elongation at 72°C, and a final extension at 72°C for 10 min**.** The forward primer of selected primer pair was fluorescently [Hexachloro-fluorescein (HEX)] labeled at M13 end. The amplification reaction conditions were performed in a total volume of 25 ul containing 100 ng DNA, 0.2 mMdNTP mix, 2.5 µM $MgCl_2$, 0.75 U *Taq* polymerase (RBC Bioscience), 0.2 µM forward primer-M13 tail, 0.3 µM M13-HEX and 0.2 µM reverse primer. The amplification reaction conditions were as follows: 5 min Pre-denaturation step at 94°C, followed by thirty cycles of 30 s at 94°C, annealing temperature depend on each primer for 45 s and 30 s elongation at 72°C. The second amplification consists of a denaturation step at 94°C for 30 s, the optimized annealing temperature was 52°C for 45 s, the extension step at 72°C for 30 s. This condition was performed for 8 cycles and a final extension at 72°C for 10 min**.**

For primer selection, the PCR products were separated on 1.2% agarose gel electrophoresis at 100 V for 25 min and visualized by ethidium bromide (0.5 µg/ml) with UV box. The primers which generate good amplification and monomorphic band were further analyzed the fragment size by separating on 3% metaphor agarose gel electrophoresis at 70 V and visualized by ethidium bromide (0.5 ug/ml) with UV box. It can distinguish DNA size between 100-600 bp. The amplification products were sent for sequencing and fragment analysis capillary electrophoresis to detect alleles using an automated DNA sequencer. DNA fragments were scored as presence (1) or absence (0) for each primer. These scores were used to calculate genetic similarity according to Nei and Li (1997) [13], from which a UPGMA cluster dendogram was constructed using NTSYS-pc 2.20e [14].

RESULTS AND DISCUSSIONS

According to the SSR analysis, the fourteen primer pairs, JaCpSSR_1, JaCpSSR_2, JaCpSSR_3, JaCpSSR_4, JaCpSSR_5, JaCpSSR_6, JaCpSSR_7, JaCpSSR_8, JaCpSSR_9, JaCpSSR_10, JaCpSSR_11, JaCpSSR_12, JaCpSSR_13 and JaCpSSR_14 were mononucleotide repeats, dinucleotide repeats and trinucleotide repeats. Ten SSRs primer-pairs were shown monomorphic band in agarose gel electrophoresis. However, four primers, JaCpSSR_2, JaCpSSR_5, JaCpSSR_10 and JaCpSSR_11were revealed polymorphic bands in 3% metaphor gel electrophoresis and they could distinguish *Jatrophacurcas* from other *Jatropha* species. These primers were used to amplify microsatellite in noncoding region of

*trn*S-*trn*R, *trn*L-*trn*F, *psb*C-*trn*S and *psb*M-*trn*D, respectively.

JaCpSSR_2, JaCpSSR_5 and JaCpSSR_11 primers generated polymorphic bands in *J. curcas*(Chiang Mai), *J. curcas* (Loey) and *J. curcas* (South Africa). However, the nucleotide sequences indicated that the difference among the samples caused by the flanking region not the SSR regions [15]. Moreover, the fragment analysis found 25 alleles different. The dendrogram generated through UPGMA method of NTSYSpc-2.20k. The genetic similarity coefficient value varied from 0.68-1.00 (Figure 1) and intraspecific of *J. curcas* ranged from 0.76-1.00. According to the study of genetic diversity of *J. Caracas*in Hainan and China are using AFLP marker revealed very low genetic variation [16] and the genetic relationship analysis in the genus *Jatropha* in Thailand using ISSR techniques also showed a high similarity in *J. curcas*[17]. In contract, Wen et al. (2010) [18] used simple sequence repeat (SSR) markers to analyze the genetic relationships among 45 accessions of *J. curcas* from germplasm collection in China. The reslt represent that *J. curcas* germplasm collection had a high level of genetic diversity.

The dendrogram show the relationship among 17 *Jatropha* accessions were resolved into three major groups. Cluster I was the largest including all of *J. curcas* which *J. curcas*1 (Chiang Mai), *J. curcas*2 (Loey) and *J. curcas*6 (Lampang) were grouped together with high similarity coefficient (1.00) accordance with geographical origin. Similarly, *J. curcas* from Lamphun and Khonkaen with the similarity index was 1.00. However, in contrast to *J. curcas* 11(Maxico) and 12 (Maxico) that were not closely related to each other because they may came from diverse origin. Several researchers in genetic diversity of *J. curcas* represented that no correlation between genetic similarity and geographic origin [19]. Group II consist of *J. integerrima*, *J. multifida* and *J. gossypifolia* and Group III was the *J. podagrica*in agreement with morphology of pollen. Four species show a distinct triangular pattern on their exine wall [20].

Table 2: List of Chloroplast SSR Polymorphic Primers Sequence

Primer	Sequences	Repeat Motif	Genomic Coordinates of SSR loci
JaCpSSR_2	F: AGAAGATAGAAGAATTAATCCATCA	(A)27, (AT)8, (AT)6, (AT)5, (AT)4, (T)10	10080 - 10500
	R: TAGAAGACCTCTGTCCTATCC		
JaCpSSR_5	F: AAAATGGGCAATCCTGAG	(A)9, (A)7, (T)10	51840 -52260
	R: TATTCTCGTCCGATTAATCAG		
JaCpSSR_10	F: TCCTCTGCAGTATTGGGCTTT	(G)9, (T)5, (T)6, (A)5	38580 - 38940
	R: TGGAACCTAACCATACATGCC		
JaCpSSR_11	F:GAAACAGAGAAAGGGGAGTTA	(A)6, (A)12, (T)8	32640 - 33130
	R: GTATCGTCCAAATACAAATTCCA		

Rows\Cols	Ja1	Ja2	Ja3	Ja4	Ja5	Ja6	Ja7	Ja8	Ja9	Ja10	Ja11	Ja12	Ja13	Ja14	Ja15	Ja16	Ja17
Ja1	1.000																
Ja2	1.000	1.000															
Ja3	0.840	0.840	1.000														
Ja4	0.840	0.840	0.760	1.000													
Ja5	0.760	0.760	0.920	0.840	1.000												
Ja6	1.000	1.000	0.840	0.840	0.760	1.000											
Ja7	0.840	0.840	0.680	0.840	0.760	0.840	1.000										
Ja8	0.760	0.760	0.920	0.840	1.000	0.760	0.760	1.000									
Ja9	0.920	0.920	0.840	0.840	0.760	0.920	0.760	0.760	1.000								
Ja10	0.840	0.840	0.920	0.760	0.840	0.840	0.680	0.840	0.840	1.000							
Ja11	0.840	0.840	0.920	0.760	0.840	0.840	0.680	0.840	0.840	0.840	1.000						
Ja12	0.920	0.920	0.840	0.920	0.760	0.920	0.760	0.760	0.920	0.840	0.840	1.000					
Ja13	0.760	0.760	0.760	0.760	0.760	0.760	0.680	0.760	0.760	0.760	0.760	0.760	1.000				
Ja14	0.760	0.760	0.760	0.760	0.760	0.760	0.680	0.760	0.760	0.760	0.760	0.760	0.920	1.000			
Ja15	0.760	0.760	0.760	0.760	0.760	0.760	0.680	0.760	0.760	0.760	0.760	0.760	0.760	0.760	1.000		
Ja16	0.760	0.760	0.760	0.760	0.760	0.760	0.680	0.760	0.760	0.760	0.760	0.760	0.760	0.760	0.760	1.000	
Ja17	0.680	0.680	0.680	0.680	0.680	0.680	0.760	0.680	0.680	0.680	0.680	0.680	0.680	0.680	0.680	0.680	1.000

Figure 1: Similarity Index of 17 Accession of *Jatropha*

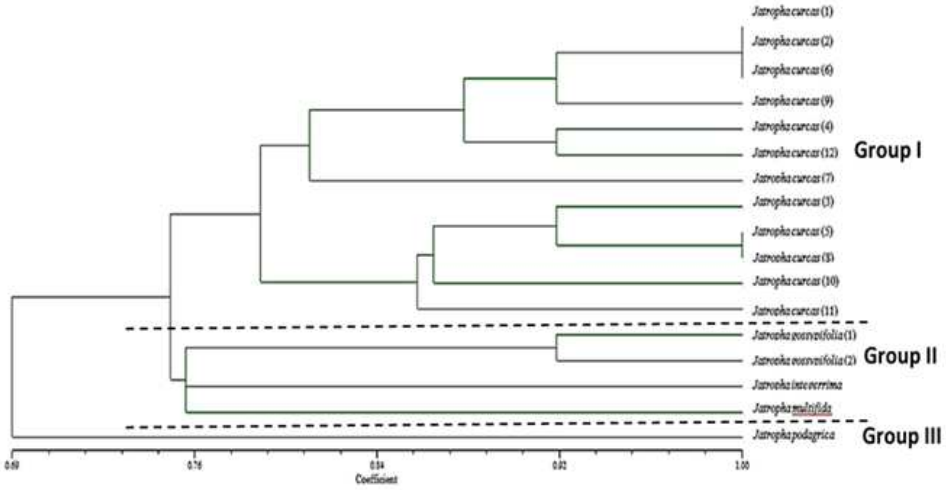

Figure 2: Dendroagram show the Relationships among the 17 Accession of *Jatropha*. The Dendrogram was Generated from Similarity Index based on UPGMA

CONCLUSIONS

Four of fourteen cpSSRs markers including JaCpSSR_2, JaCpSSR_5, JaCpSSR_10 and JaCpSSR_11 were used to amplify 17 *Jatropha* accessions which were twelve of *J. curcas* and five of other *Jatropha* species. They were resolved into three major groups these newly developed cpSSRs have not produced polymorphic bands among intraspecific. However, these markers can further use for selective marker in studying the relationship and developing high yield product cultivars.

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